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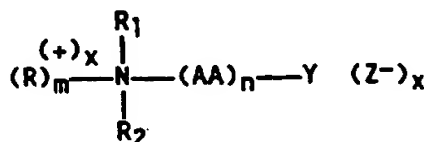
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(54) **Peptide skeletal muscle relaxants.**

(57) A method of achieving muscle relaxation comprising administering a polypeptide represented by the formula:



wherein m is 0 or 1, n is 3-5, x is 0 or 2; R is lower alkyl, R₁ and R₂ are independently hydrogen, lower alkyl, allyl, propargyl, aryl lower alkyl, cyclo-lower alkyl lower alkyl and -COOR₃ or R plus one or both of R₁ and R₂ and the attached nitrogen form a 5- to 7-member heterocyclic ring, at least one AA is a basic amino acid, at least one AA is a lipophilic amino acid, Y is -NH₂ or -OR₄, R₃ is t-butyl, benzyl or fluorenylmethyl, and R₄ is lower alkyl, aryl lower alkyl and N-(lower alkyl)piperidyl lower alkyl, Z is a pharmaceutically acceptable anion and, wherein m and x are 0, pharmaceutically acceptable acid addition salts thereof. Diquaternary compounds within the above formula are novel.

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This invention relates to certain tri-, tetra- and pentapeptides useful as nondepolarizing muscle relaxants.

Background of the Invention

Polypeptides comprised of a relatively low number of amino acid residues, yet which possess therapeutic activity, are known in the art. For example, Okai Japanese Patent No. 63-215697 (unexamined) discloses polypeptides containing at least five amino acid residues which are stated to have muscle relaxant activity. The type of muscle relaxant activity possessed by the disclosed polypeptides is not given.

Gieger, et al., U.S. Patent No. 4,623,715, discloses polypeptides containing four or more amino acid residues, preferably pentapeptides and hexapeptides, which are useful as mood-elevators, antidepressants and anxiolytic agents.

Gormley, U.S. Patent No. 4,421,744, discloses peptides and pseudo-peptides of the formula $(R)_2N-A-B-D-E-F-X$ wherein A and F are amino acid residues, and each of B, D and E can be an amino acid residue or a valency bond. These compounds are active as opiate receptor agonists.

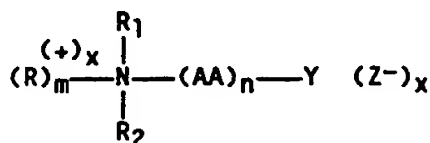
Wilkinson, U.S. Patent No. 4,254,106, discloses N,N-dialkyl, i.e. N-terminal tertiary, polypeptides containing at least five amino acid residues which are useful as morphine agonists.

Kahn et al. Tetrahedron Letters, Vol. 27, No. 40, pp 4841-4844, (1986), and Najjar, U.S. Patent No. 3,778,426, disclose polypeptides which mimic the activity of the naturally occurring tetrapeptide tuftsin which is an immunostimulant.

In accordance with the present invention, it has been found that certain polypeptides, i.e. tri-, tetra- and pentapeptides, possess significant nondepolarizing muscle relaxant activity, thus making them useful therapeutically as skeletal muscle relaxants.

Summary of the Invention

The present invention pertains to a method of producing a skeletal muscle relaxing effect in a mammal by the administration of an effective amount of a compound selected from those represented by the formula:



wherein:

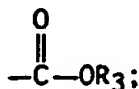
m is 0 or 1;

n is 3, 4 or 5;

x is 0 or 2;

R is lower alkyl; and

R_1 and R_2 are independently selected from the group consisting of hydrogen, lower alkyl, allyl, propargyl, aryl lower alkyl, cyclo-lower alkyl lower alkyl and



or R plus one or both of R_1 and R_2 , together with the nitrogen to which they are attached, form a heterocyclic ring having 5 to 7 member atoms;

at least one AA is a basic amino acid, except when R_4 is N-(lower alkyl)-substituted piperidyl lower alkyl;

at least one AA is a lipophilic amino acid;

Y is $-NH_2$ or $-OR_4$;

Z^- is a pharmaceutically acceptable anion;

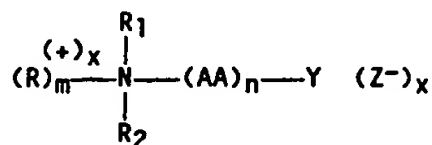
R₃ is t-butyl, benzyl or fluorenylmethyl;

R₄ is lower alkyl, aryl lower alkyl and N-(lower alkyl)-substituted piperidyl lower alkyl; and wherein m and x are 0, pharmaceutically acceptable acid addition salts thereof.

Certain of the subject polypeptides are novel compounds.

Detailed Description of the Invention

The polypeptides useful in the therapeutic method of the present invention are those represented by the formula:



wherein:

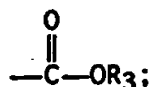
m is 0 or 1;

n is 3, 4 or 5;

x is 0 or 2;

R is lower alkyl;

R₁ and R₂ are independently selected from the group consisting of hydrogen, lower alkyl, allyl, propargyl, aryl lower alkyl, cyclo-lower alkyl lower alkyl and



or R plus one or both of R₁ and R₂, together with the nitrogen to which they are attached form a heterocyclic ring having 5 to 7 member atoms;

at least one AA is a basic amino acid selected from the group consisting of DAB, Orn, Lys, Arg, homo-Arg, DAB(γ-N-(R)_mR₁R₂), Orn(δ-N-(R)_mR₁R₂) and Lys(ε-N-(R)_mR₁R₂);

at least one AA is a lipophilic amino acid selected from the group consisting of Ala, Val, Leu, Ile, Phe, Tyr, Pro, Tic, 1-Naphthala, 2-Naphthala, homo-Phe, AcOPro and Glu(γ-Me), or two of the lipophilic AA are cystine;

Y is -NH₂ or -OR₄;

Z⁻ is a pharmaceutically acceptable anion;

R₃ is selected from the group consisting of t-butyl, benzyl or fluorenylmethyl; and

R₄ is selected from the group consisting of lower alkyl, aryl lower alkyl and N-(lower alkyl)-piperidyl lower alkyl, with the proviso that, when R₄ is N-(lower alkyl)-piperidyl lower alkyl, a basic amino acid is not present;

wherein m is 1, x is 2, and said basic amino acid is selected from the group consisting of DAB(γ-N-(R)_mR₁R₂), Orn(δ-N-(R)_mR₁R₂) and Lys(ε-N-(R)_mR₁R₂) or R₄ is n-(lower alkyl)-piperidyl lower alkyl, and wherein m is 0, pharmaceutically acceptable acid addition salts thereof.

In accordance with the present invention, the term "lower alkyl" is a branched- or unbranched-hydrocarbon radical containing from 1 to 7 carbon atoms. Preferred lower alkyl groups in accordance with the subject invention are methyl and ethyl. The term "cyclo-lower alkyl", as utilized herein, means cyclic alkyl groups containing from 3 to 6 carbon atoms. The term "aryl" as utilized herein is intended to mean phenyl or naphthyl which may be unsubstituted or substituted with up to three substituents selected from the group consisting of halogen, halogenated lower alkyl lower alkyl, hydroxy and lower alkoxy. Preferred aryl groups include phenyl, 1-naphthyl and 2-naphthyl.

The amino acids which form the subject polypeptides will be referred to herein by their conventional abbreviations for the sake of brevity. Such abbreviations are well known to those skilled in the art. The primary abbreviations are given below:

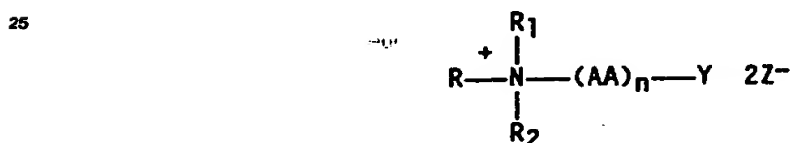
Alanine

- Ala

	Leucine	- Leu
	Phenylalanine	- Phe
	Tyrosine	- Tyr
	Arginine	- Arg
5	Ornithine	- Orn
	Glutamic Acid	- Glu
	2,4-Diaminobutyric Acid	- DAB
	4-Acetoxypoline	- 4-AcOPro
	Tetrahydroisoquinoline Carboxylic Acid	- Tic
10	Glutamic Acid Gamma Methyl Ester	- Glu(γ -Me)
	1- and 2-Naphthylalanine	- 1- and 2-Naphthala
	Gamma-aminobutyric acid	- GABA
	Valine	- Val
	Isoleucine	- Ile
15	Tryptophan	- Trp
	Proline	- Pro
	Cystine	- Cys
	Lysine	- Lys
	Nipecotic Acid	- Nip

20 Unless it is specified herein that the amino acid moieties which make up the subject peptides are in the D-form or the L-form, both forms are intended.

Novel compounds within the formula are the diquaternary polypeptides, i.e. those compounds represented by the formula:



wherein:

n is 3, 4 or 5;

R is lower alkyl; and

35 R_1 and R_2 are independently selected from the group consisting of lower alkyl, allyl, propargyl, aryl lower alkyl, cyclo-lower alkyl lower alkyl and



45 or R plus one or both of R_1 and R_2 , together with the nitrogen to which they are attached form a heterocyclic ring having 5 to 7 member atoms;

at least one AA is a basic amino acid selected from the group consisting of DAB(γ -N- RR_1R_2), Orn(δ -N- RR_1R_2) and Lys(ϵ -N- RR_1R_2);

50 at least one AA is a lipophilic amino acid selected from the group consisting of Ala, Val, Leu, Ile, Phe, Tyr, Pro, Tic, 1-Naphthala, 2-Naphthala, homo-Phe, AcOPro and Glu(γ -Me), or two of the lipophilic AA are cystine;

Y is $-NH_2$ or $-OR_4$;

R_3 is selected from the group consisting of t-butyl, benzyl or fluorenylmethyl;

60 R_4 is selected from the group consisting of lower alkyl, aryl lower alkyl and N-(lower alkyl)-piperidyl lower alkyl with the proviso that, wherein R_4 is N-(lower alkyl)-piperidyl lower alkyl, a basic amino acid is not present; and

Z^- is a pharmaceutically acceptable anion.

These compounds also represent a preferred group of compounds within the scope of the present invention. Also, among the muscle relaxants of the present invention, the tripeptides are a preferred group

of compounds.

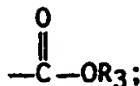
Other preferred compounds within the scope of the present invention are those in the above formula wherein n is 3, R₁ and R₂ are independently selected from the group consisting of methyl, ethyl, allyl, benzyl and cyclopropyl methyl. Wherein R plus one or both of R₁ and R₂, together with the nitrogen to which they are attached, form a 5- to 7-member heterocyclic ring, such rings may contain additional hetero atoms, i.e. N, O or S. Examples of suitable heterocyclic rings include pyrrolidine, piperidine, hexamethyleneimine, piperazine, morpholine, and the like.

A particularly preferred group of novel diquaternary peptides within the scope of the present invention are those compounds of the above formula wherein R₁ and R₂ are independently selected from the group consisting of methyl, ethyl, allyl, benzyl and cyclopropyl methyl, or R plus one or both of R₁ and R₂, together with the nitrogen to which they are attached, form a 5- to 7-member heterocyclic ring, the basic amino acids are selected from the group consisting of DAB(γ-N-RR₁R₂), Orn(δ-N-RR₁R₂) and Lys(ε-N-RR₁R₂), the lipophilic amino acids are selected from the group of Leu, Phe, Tyr and Pro, and R₄ is methyl, ethyl or benzyl.

Another preferred group of novel compounds within the scope of the present invention are those compounds represented by the formula given above wherein m is zero in the peptide chain, and one in the side chain, i.e. wherein a basic amino acid moiety is, for example,

DAB(γ-N-RR₁R₂),

n is 3 and one of R₁ and R₂ in the peptide chain is hydrogen and the other is

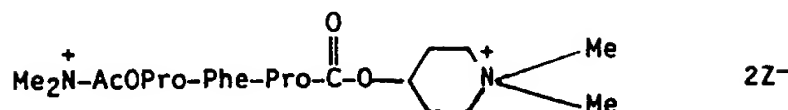
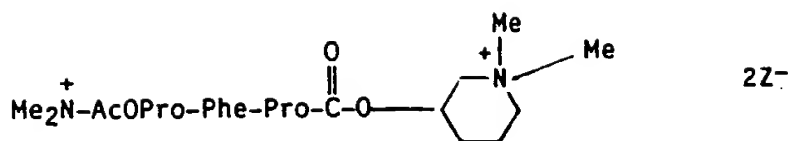


R₁ and R₂ in the side chain are as defined above with the exception that neither is hydrogen, the basic amino acids are selected from the group consisting of DAB(γ-N-RR₁R₂), Orn(δ-N-RR₁R₂) and Lys(ε-N-RR₁R₂), the lipophilic amino acids are selected from the group consisting of Leu, Phe, Tyr, Tic, 1-Naphthala and homo-Phe, R₃ is t-butyl and R₄ is methyl, ethyl or benzyl. The abbreviation "Boc" used in reference to this group of compounds indicates t-butyloxycarbonyl. It will be appreciated that compounds within this latter group wherein R₁ and R₂ are other than hydrogen are novel compounds.

Specific preferred polypeptides falling within the preceding two groups are the following:

Quaternary Polypeptides

Me₃N⁺-Phe-Leu-Lys(ε-N-Me₃)OMe 2Z⁻ (all AA D-form)
 Me₃N⁺-Phe-Leu-Orn(δ-N-Me₃)OMe 2Z⁻
 Me₃N⁺-Glu-(γ-Me)-Phe-Lys(ε-N-Me₃)OMe 2Z⁻
 Me₂N⁺-Pro-Phe-Orn(δ-N-Me₃)OMe 2Z⁻
 Boc-Lys(ε-N-Me₃)-Phe-Lys(ε-N-Me₃)OMe 2Z⁻
 Boc-Lys(ε-N-Me₃)-Phe-Lys(ε-N-Me₃)OMe 2Z⁻ (all AA D-form)
 Boc-Lys(ε-N-Me₃)-D-Phe-Lys(ε-N-Me₃)OMe 2Z⁻
 Boc-Lys(ε-N-Me₃)-2-Naphthala-Lys(ε-N-Me₃)OMe 2Z⁻
 Boc-Lys[ε-Me-N(CH₂)₅]-Phe-Lys[ε-Me-N(CH₂)₅]OMe 2Z⁻



Non-Quaternary Polypeptides

Pro-(γ -N-Me₂)-DAB-PheOMe

MePro-Pro-Lys(ϵ -N(CH₂)₅-)OMe

20 Boc-Lys-Tic-Lys-OMe

Boc-homo-Arg-Phe-homo-ArgOMe

Boc-Lys-Phe-LysOMe

Boc-Lys(ϵ -N(CH₂)₅-)-Phe-Lys(ϵ -N(CH₂)₅-)OMe

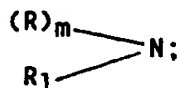
25 In still another preferred group of compounds within the scope of the present invention, m is 1, n is 3, R₁ and R₂ are selected from the group consisting of methyl, ethyl, allyl, benzyl and cyclopropyl methyl, the only amino acid moieties present are lipophilic amino acids selected from the group consisting of Leu, Phe, Pro, Tyr and AcOPro, Y is OR₄ and R₄ is N substituted piperidyl lower alkyl.

The pharmaceutically acceptable anions designated by Z⁻ in the above formula include, for example, 30 inorganic anions such as the chloride, bromide, sulfate and the like, and organic anions such as the acetate, trifluoroacetate oxalate, citrate, benzene sulfonate, tartrate and the like. Preferred anions are the chloride, the iodide, the trifluoroacetate and the benzene sulfonate.

Generally, the polypeptide skeletal muscle relaxants of the present invention may be prepared by 35 solution phase peptide synthetic procedures analogous to those described hereinafter or methods known to those skilled in the art. For example, carboxylic moieties such as N- α -carbobenzyloxy (Cbz), N- α -fluorenylmethyloxycarbonyl(Fmoc), and N- α -t-butyloxycarbonyl(Boc) or substituted amino acid derivatives having suitable side chain protecting groups, if necessary, may be condensed with the amino functionality of a suitably protected amino acid or peptide using conventional coupling protocols such as dicyclohexyl- 40 carbodiimide (DCC), N-diethylaminopropyl-N'-cyclohexylcarbodiimide (EDCC) or 1-hydroxybenzotriazole (HOBt) in methylene chloride or dimethylformamide. Such coupling reactions are described, for example, in Meienhofer-Gross, *The Peptides*, Academic Press, Vol. 1, (1979) or Bodanszky-Bodanszky, *The Practice of Peptide Synthesis*, Springer-Verlag, (1984).

Following coupling reaction completion, the protectant moieties are removed as follows. The N- α -Boc 45 moiety may be selectively removed with 50% trifluoroacetic acid (v/v) in methylene chloride. Neutralization of the resultant trifluoroacetate salt may be accomplished with a slight excess of triethylamine or diisopropylethylamine in methylene chloride. In the case of the N- α -Cbz moiety, selective removal is accomplished using hydrogen gas and a catalyst such as 5-10% palladium on carbon in a lower alkanol solvent such as methanol, ethanol or 2-propanol. Selective removal of the N- α -Fmoc moiety may be 50 accomplished using 20% piperidine (v/v) in methylene chloride.

There is also provided in accordance with the invention a process for the manufacture of those of the 55 compounds of formula I which contain the groups:



DAB(γ -N-(R)_mR₁);
 Orn(δ -N-(R)_mR₁); and
 Lys(ϵ -N-(R)_mR₁);

- 5 where m = 1 and R₁ and R₂ are independently selected from the group consisting of lower alkyl, aryl lower alkyl and cyclo-lower alkyl lower alkyl, and pharmaceutically acceptable salts thereof which comprises reacting the corresponding compound containing the group NH₂-(AA)_n-, generated either in situ by conventional deblocking methodology or from an isolated intermediate by reaction with the appropriate aldehyde or ketone and an alkali metal hydride reducing agent or hydrogen gas and a catalyst such as 5-10% palladium on carbon. The aldehyde or ketone may be, for example, formaldehyde, acetaldehyde, benzaldehyde, cyclopropane carboxaldehyde, glutaraldehyde, or acetone. The alkali metal hydride may be, for example, an alkali metal borohydride such as sodium cyanoborohydride. The process is conveniently carried out in a suitable solvent, such as methanol, optionally together with acetic acid or 1-hydroxyethyl-piperazine ethanesulfonic acid (HEPES), at ambient temperature. The methods mentioned are described, for example, in Gormley, U.S. Patent No. 4,421,744 (1983).

The compounds of formula I which contain the groups:

- (R_m)R₁R₂N-;
 DAB(γ -N-(R)_mR₁R₂);
 20 Orn(δ -N-(R)_mR₁R₂); and
 Lys(ϵ -N-(R)_mR₁R₂),

- where m = 1; and R₁ and R₂ are independently selected from the group consisting of lower alkyl, allyl, propargyl, aryl lower alkyl and cyclo-lower alkyl lower alkyl or R plus one or both of R₁ and R₂, together with the nitrogen to which they are attached, form a heterocyclic ring having 5 to 7 member atoms, and pharmaceutically acceptable salts thereof are prepared by reacting a compound containing the group:



- with a compound of the formula R₂Hal wherein R₂ has the meaning stated above and Hal is a halogen atom in a suitable solvent for example, methanol, ethanol, acetonitrile and dimethylformamide.

- A compound containing the group NH₂-(AA)- may be reacted with a compound of the formula R₁Hal or R₂Hal wherein R₁, and R₂ and Hal have the meanings stated above, in the presence of an acid-binding agent. A suitable acid-binding agent is, for example, an alkali metal carbonate or bicarbonate such as potassium carbonate or sodium bicarbonate. The process is conveniently carried out in a suitable solvent such as, for example, methanol, ethanol, acetonitrile or dimethylformamide. This method is described, for example, in Benoiton-Chen, *Proced. 14th Europ. Pept. Symp.*, (1976), p. 149.

- The compounds of the present invention are administered parenterally, i.e. by intravenous, intramuscular or subcutaneous administration, in the form of an aqueous solution. The nonquaternary polypeptides are present in such preparations in the form of their pharmaceutically acceptable acid addition salts. Suitable carriers for the acid addition salts include isotonic water, sterile water for injection (USP), alone or in combination with other solubilizing agents such as ethanol, propylene glycol, or other conventional solubilizing agents known to those skilled in the art. A preferred carrier is an isotonic aqueous solution of the inventive compound.

- Suitable acid addition salts include, for example, inorganic acid salts such as hydrochloric, hydrobromic, sulfuric, nitric, phosphoric, perchloric acid salts and the like; and organic acid salts such as acetic, trifluoroacetic, propionic, oxalic, hydroxyacetic, methoxyacetic, 2-hydroxypropanoic, 2-oxopropanoic, propanedioic, 2-hydroxybutanedioic, benzoic, 2-hydroxybenzoic, 4-amino-2-hydroxybenzoic, 3-phenyl-2-propanoic, alpha-hydroxyphenylacetic, methanesulfonic, ethanesulfonic, benzenesulfonic, toluenesulfonic, cyclohexanesulfamic, succinic, tartaric, citric, maleic, fumaric acid salts and the like. Preferred acid addition salts include chloride, trifluoroacetate, iodide and benzenesulfonate. These acid addition salts can be prepared by conventional methods, such as by treatment of the free base of the subject compound with the appropriate acid.

Sterile solutions or suspensions of the compounds of the present invention preferably contain at least

about 0.1% by weight of the active compound, but this amount may be varied to as much as about 50% by weight. The exact amount of the subject compound present in such compositions is such that a suitable dosage level will be obtained. Preferred compositions and preparations according to the present invention are prepared so that a parenteral dosage unit contains from between about 150 to about 1000 milligrams of a compound of formula I.

The sterile solutions or suspensions prepared in accordance with the subject invention may also include the following adjuvants: a sterile diluent, such as water for injection, saline solution, fixed oils, polyethylene glycol, glycerine, propylene glycol, or other synthetic solvent; antibacterial agents, such as benzyl alcohol or methyl paraben; antioxidants, such as ascorbic acid or sodium metabisulfite, chelating agents such as ethylenediaminetetraacetic acid (EDTA); buffers, such as acetates, citrates or phosphates; and agents for the adjustment of tonicity, such as sodium chloride or dextrose. The parenteral preparations may be dispensed in ampules, disposable syringes, or multiple dose vials made of glass or plastic.

The compounds of the present invention can be administered to mammals, e.g., animals or humans, in amounts effective to provide the desired muscle relaxant therapeutic effect. Since the activity of the compounds and the degree of the desired therapeutic effect vary, the dosage level of the compound employed will also vary. The actual dosage administered will also be determined by such generally recognized factors as the body weight of the patient and the individual hypersensitiveness of the particular patient. Thus, the unit dosage for a particular patient (man) can be as low as about 2 mg/kg, which the practitioner may titrate to the desired effect.

The polypeptides of the present invention are skeletal muscle relaxants of the competitive or non-depolarizing type (curariform). This type of muscle relaxant is preferred over the depolarizing agents, such as succinylcholine chloride, because they are easier to control. Nondepolarizing skeletal muscle relaxants antagonize the neurotransmitter action of acetylcholine by binding competitively with cholinergic receptor plates on the motor end plate. Most known nondepolarizing skeletal muscle relaxants, such as pancuronium bromide, cecurium besylate and vecuronium bromide, have an intermediate duration of therapeutic activity. The subject compounds are advantageous in comparison therewith since, in addition to having a very rapid onset of action, they possess a short duration of activity. These properties are particularly useful as an adjunct to general anesthesia. The preferred polypeptides of the present invention are useful to facilitate short medical procedures such as endotracheal intubation, and for skeletal muscle relaxation during surgery or mechanical ventilation. These therapeutic indications are considered to be unexpected since, to Applicants' knowledge, there are no other polypeptides possessing this type of activity.

The following Examples illustrate this invention, it being understood that the invention is in no way intended to be limited to the details described therein. In the Examples, all parts and percentages are on a weight basis and all temperatures are in degrees Celsius, unless otherwise stated.

Example 1

Tris-trifluoroacetate salt of N,N,N-Trimethyl-D-phenylalanyl-D-leucyl-D-(N- ϵ -trimethyl)lysine methyl ester.

A stirred solution of D-(N- ϵ -Cbz)lysine (1.0 g., 3.57 mmol) in methanol (25 ml) was cooled to 0° and treated dropwise with acetyl chloride (4.4 ml, 62 mmol). The ice bath was removed and, after stirring for 17 hours at room temperature, the reaction mixture was concentrated and then dried in vacuo to give 1.24 g. of a white, foamy solid. There was obtained D-(N- ϵ -benzyloxycarbonyl)lysine methyl ester, essentially pure by HPLC, gradient system: Buffer B, 0 - 100% in 30 minutes, flow rate 1 ml/min (Buffer A, 0.1% TFA in H₂O; Buffer B, Acetonitrile), on a RP C-18 Vydac column and UV detection at 214 nm. A single peak eluting at R_t = 15.59 min was observed.

The resulting product (1.17 g., 3.3 mmol) was dissolved in methylene chloride (5 ml) and DMF (10 ml) and cooled to 0° in an ice water bath. Triethylamine (0.5 ml, 3.6 mmol), 1-hydroxybenzotriazole (0.45 g., 3.3 mmol), N- α -t-butyloxycarbonyl-D-leucine (0.77 g., 3.3 mmol) and dicyclohexylcarbodiimide (0.687 g., 3.3 mmol) were added to the cooled solution. Stirring was continued for 7 hours during which the reaction mixture was allowed to warm to room temperature. The mixture was filtered and the precipitate washed with THF. The filtrate was concentrated in vacuo, and the residue taken up in EtOAc (200 ml) and washed with aqueous NaHCO₃, 1M HCl, aqueous NaHCO₃ and brine. The organic fraction was dried over MgSO₄ and concentrated to yield N- α -t-butyloxycarbonyl-D-leucyl-D-(N- ϵ -benzyloxycarbonyl)lysine methyl ester as an amorphous residue weighing 1.58 g. HPLC showed only one peak at R_t = 24.0 min.

The ester prepared above (1.58 g., 3.12 mmol) was dissolved in methylene chloride (20 ml) and cooled in an ice water bath. Anisole (0.8 ml) and trifluoroacetic acid (TFA) (16 ml) were added, the reaction mixture was stirred at 0° for 2 hours and then concentrated to an oil. The oil was dissolved in water (150 ml) and

washed with Et₂O three times. The aqueous layer was lyophilized to give 0.51 g. of a white powder. The organic layer was backwashed with water and the new aqueous layer lyophilized to yield an additional 0.28 g of product. HPLC showed only one peak at R_t = 17.66 min.

The resulting product (0.51 g., 0.98 mmol) was dissolved in CH₂Cl₂ (5 ml) and DMF (5 ml), cooled in an ice water bath and treated with triethylamine (0.14 ml, 1.05 mmol). α-Cbz-D-phenylalanine (0.29 g., 0.98 mmol), 1-hydroxybenzotriazole (0.13 g., 0.98 mmol) and dicyclohexylcarbodiimide (0.20 g., 0.98 mmol) were added to the stirred solution. The reaction mixture was allowed to warm to room temperature and stirred for a total of 17 hours. The solid product was filtered off, washed with THF and the filtrate concentrated. EtOAc (200 ml) was added to the filtrate which was then washed with aq NaHCO₃, 1 M HCl, aq NaHCO₃ and brine. The organic layer was dried over MgSO₄ and concentrated in vacuo to give 0.64 g. of N-α-benzyloxycarbonyl-D-phenylalanyl-D-leucyl-D-(N-ε-benzyloxycarbonyl)lysine methyl ester as a white solid. TLC with 30:1 CHCl₃: MeOH showed only one product (R_f = 0.6).

To a solution of the resulting methyl ester (673 mg, 0.98 mmol) in methanol (100 ml) was added 10% Pd/C (210 mg in 2 ml of water). The mixture was hydrogenated in a Parr hydrogenation apparatus (P=50) for 7 hours, purged with N₂ and the catalyst filtered off. The filtrate was concentrated in vacuo, taken up in methanol and concentrated again to give 410 mg of D-phenylalanyl-D-leucyl-D-lysine methyl ester as a white powder. HPLC indicated a major product at R_t = 13.14 min. together with a few minor impurities. The material was used as is.

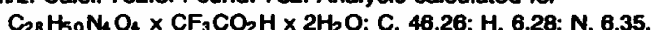
The resulting product (0.41 g., 0.97 mmol) was dissolved in methanol (15 ml). To the solution was added NaHCO₃ (0.40 g., 4.76 mmol) and methyl iodide (8 ml, 96 mmol) and the reaction mixture was stirred at room temperature for 72 hours. The mixture was concentrated in vacuo to give 1.4 g. of a yellow solid. HPLC showed three peaks at R_t = 3.09 min. (HI), 13.39 min. (Product), and 16.67 min. (impurity).

The product was purified by RP-HPLC using Vydac C-18 one-inch column; gradient system: Buffer B, 0-100% in 30 min., flow rate 20 ml/min., UV detection at 220 nm (Buffer A, 0.1% TFA/H₂O; Buffer B, Acetonitrile). The pooled fractions were lyophilized to give 0.20 g. of tris-trifluoroacetate salt of N,N,N-trimethyl-D-phenylalanyl-D-leucyl-D-(N-ε-trimethyl)lysine methyl ester as a white powder, pure by analytical HPLC, R_t = 13.39 min.

The fast atom bombardment (FAB) MS of the product was consistent with the tris-trifluoroacetate salt of the desired product:



at m/z. Calc.: 732.8. Found: 732. Analysis calculated for



Found: C, 46.31; H, 5.97; N, 5.93.

Example 2

Methyl ester of N-α-t-butyloxycarbonyl-α-S-[4-[1-(1-methyl)piperidinium]butyl]glycyl-L-phenylalanyl-α-S-[4-[1-(1-methyl)piperidinium]butyl]glycine tris-trifluoroacetate.

A stirred solution of the methyl ester of (N-ε-benzyloxycarbonyl)-L-lysine hydrochloride (2.21 g., 6.7 mmol), N-α-t-butyloxycarbonyl-L-phenylalanine (2.0 g., 6.7 mmol), 1-hydroxybenzotriazole (HOBt) (1.81 g., 13.4 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCl x HCl) (2.57 g., 13.4 mmol) in CH₂Cl₂ (30 ml), was cooled to 5° in an ice bath. Triethylamine (2.19 g., 21.6 mmol) was added via syringe maintaining the temperature at 5°. After stirring for 2 hours, the reaction mixture was allowed to come to room temperature. The reaction mixture was stirred for 18 hours, washed with saturated NaHCO₃ solution, 1M HCl and brine and dried over magnesium sulfate. After removal of solvent in vacuo, the residue was triturated with ether/hexane to afford the methyl ester of N-α-t-butyloxycarbonyl-L-phenylalanyl-L-(N-ε-benzyloxycarbonyl)lysine as a white solid (3.2 g.). The structure was supported by NMR and IR.

The product (0.83 g., 1.57 mmol) was dissolved in methylene chloride (20 ml), cooled to 5° in an ice bath and treated with trifluoroacetic acid (TFA) (5 ml). The reaction mixture was stirred at room temperature for 1 hour, concentrated in vacuo and the residue triturated with diethyl ether to give an amorphous solid (0.85 g.). The NMR was consistent with the desired structure.

A solution of the above product (0.85 g., 1.53 mmol) in methylene chloride (10 ml) was cooled in an ice bath and treated with triethylamine (0.15 g., 1.53 mmol). After 30 minutes, the mixture was treated with N-α-t-butyloxycarbonyl-(N-ε-benzyloxycarbonyl)lysine (0.60 g., 1.53 mmol), 1-hydroxybenzotriazole (0.21 g., 1.53 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (WSCl x HCl) (0.29 g., 1.53 mmol).

Triethylamine (0.15 g., 1.53 mmol) was added via syringe while maintaining the temperature of the reaction mixture at 5°. Stirring was continued for 2 hours after which the reaction mixture was allowed to come to room temperature. The reaction mixture was stirred for 18 hours, washed with saturated NaHCO₃ solution, 1M HCl, and brine, and dried over magnesium sulfate. After removal of solvent in vacuo, the residue was triturated with ether/hexane to afford the methyl ester of N- α -t-butyloxycarbonyl-(N- ϵ -benzyloxycarbonyl)-L-lysyl-L-phenylalanyl-L-(N- ϵ -benzyloxycarbonyl) lysine as a white amorphous solid (0.96 g.). The solid was dissolved in a minimum amount of diethyl ether and loaded onto a column of silica gel (50 X 5 cm). The column was eluted with diethyl ether/hexane (1/1) to isolate a less polar impurity followed by ether/hexane (4/1) to afford the fully protected tripeptide derivative. The pooled fractions were evaporated in vacuo to give the product as a white solid. The NMR was consistent with the desired structure.

To a solution of the methyl ester of N- α -t-butyloxycarbonyl-(N- ϵ -benzyloxycarbonyl)-L-lysyl-L-phenylalanyl-(N- ϵ -benzyloxycarbonyl)-L-lysine (0.93 g., 1.15 mmol) in methanol (40 ml) and water (4 ml) contained in a Parr hydrogenation bottle was added 0.30 g. of 10% palladium on carbon. The resulting suspension was placed under hydrogen at 50 psi and shaken for 21 hours. The reaction mixture was filtered through celite and the filtrate concentrated to give the methyl ester of N- α -t-butyloxycarbonyl-L-lysyl-L-phenylalanyl-L-lysine bis-trifluoroacetate salt. The product was purified by RP-HPLC employing an 0.1% aqueous trifluoroacetic acid/acetonitrile linear gradient. The pooled fractions were lyophilized to give the product as a hygroscopic white solid (0.67 g.). The ¹H-NMR is consistent with the desired structure. FAB mass spec. [M+H]⁺ m/z calc'd: = 535.7. Found: 537. Anal. calcd. for C₂₇H₄₅N₅O₆ x 2CF₃CO₂H x 2H₂O; C, 46.56; H, 6.43; N, 8.76. Found: C, 46.83; H, 5.76; N, 8.53.

To a stirred solution of the above product (300 mg, 0.39 mmol) in acetonitrile (10 ml) was added 10 ml of 100 mM [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) as buffer and 6 drops of bromocresol green as indicator, followed by 0.2 ml glutaric dialdehyde (50% wt. aqueous solution, 1.10 mmol) and sodium borohydride (400 mg., 10.6 mmol). The reaction mixture was stirred at room temperature for 3 hours maintaining the pH above 5.4. The solvent was removed in vacuo, the residue dissolved in 50 ml of methylene chloride and washed several times with 5% NaHCO₃. The organic phase was dried over MgSO₄, filtered and concentrated. The residue was purified by RP-HPLC using a linear gradient of 0.1% aqueous trifluoroacetic acid acetonitrile. The pooled fractions were lyophilized to give the methyl ester of N- α -t-butyloxycarbonyl- α -S-[4-(1-piperidino)butyl]glycyl-L-phenylalanyl- α -S-[4-(1-piperidino)butyl] glycine tris-trifluoroacetate as a white solid (95 mg). The ¹H-NMR was consistent with the desired structure. FAB mass spec. [M+H]⁺ = 672. Anal. calcd. for C₃₇H₆₁N₅O₆ x 3CF₃CO₂H : C, 50.93; H, 6.36; N, 6.91. Found: C, 50.81; H, 6.60; N, 7.33.

To a stirred solution of the methyl ester of N- α -t-butyloxycarbonyl- α -S-[4-(1-(piperidino)butyl]glycyl-L-phenylalanyl- α -S-[4-(1-piperidino)butyl] glycine (300 mg, 0.45 mmol), prepared by passing a solution of the tris-trifluoroacetate salt in methanol (5 ml) through a short column of potassium carbonate, was added methyl iodide (5 ml, 80.3 mmol). After stirring at room temperature for 48 hours, the reaction mixture was concentrated and the residue purified by RP-HPLC employing a 0.1% aqueous trifluoroacetic acid/acetonitrile linear gradient. The pooled fractions were lyophilized to give the methyl ester of N- α -t-butyloxycarbonyl- α -{4-[1-(1-methyl)piperidinium]butyl]glycyl-L-phenylalanyl- α -S-[4-[1-(1-methyl)piperidinium]butyl]glycine tris-trifluoroacetate as a white solid (280 mg). The ¹H-NMR was consistent with the desired structure. FAB mass spec. [M+2CF₃CO₂]⁺ = 928. Anal. calcd. for C₃₉H₆₇N₅O₆ x CF₃CO₂ x H₂O : C, 50.99; H, 6.66; N, 6.61. Found C, 51.01; H, 6.63; N, 6.65.

Example 3

Tetrakis-trifluoroacetate salt of 1,1,-dimethyl-4-piperidyloxy ester of N,N-dimethyl-trans-4-acetoxy-L-prolyl-L-phenylalanyl-L-proline.

A suspension of 10% palladium on carbon in water (25 ml) was added to a suspension of trans-4-hydroxy-L-proline (6.576 g., 50.10 mmol) in methanol (100 ml) and 37% aqueous formaldehyde (16 ml, 213.5 mmol) in a Parr bottle. The bottle was pressurized to 50 psi with hydrogen and shaken overnight. The catalyst was removed by filtration through a pad of celite which was washed thoroughly with methanol (100 ml). Evaporation of the methanol and excess formaldehyde yielded N-methyl-trans-4-hydroxy-L-proline as a white solid which was recrystallized from methanol/diethyl ether to give 6.425 g. of white needles, m.p. 236°-238° (decomposition with gas evolution).

A suspension of the above product (7.286 g., 50.1 mmol) in glacial acetic acid (75 ml) was stirred and cooled in an ice bath during the dropwise addition of acetyl chloride (34.0 ml 478.2 mmol) in 25 min. The resulting thick cream suspension was allowed to warm to ambient temperature and stirred 7 hours.

Evaporation of the glacial acetic acid and excess acetyl chloride gave a solid residue which was suspended in diethyl ether and filtered. The white solid was washed liberally with diethyl ether and dried to yield N-methyl-trans-4-acetoxy-L-proline hydrochloride (11.10 g.) mp 199°-202° (decomposition). The product was characterized by IR, NMR and MS. Anal. calcd. for $C_8H_{13}NO_4 \times HCl$: C, 42.96; H, 6.31; N, 6.26. Found: C, 42.96; H, 6.42; N, 6.18.

Triethylamine (2.0 ml, 15.0 mmol) was added dropwise to a suspension of N- α -t-butoxycarbonyl-L-phenylalanine (1.325 g., 5.0 mmol) and L-proline benzyl ester hydrochloride in methylene chloride (20 ml), stirred and cooled to 0°. 1-Hydroxybenzotriazole (0.975 g., 5.0 mmol) and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (1.032 g., 5.40 mmol) were added to the resulting clear solution. The reaction mixture was allowed to warm to ambient temperature overnight. Methylene chloride (50 ml) was added and the organic solution washed sequentially with 5% $NaHCO_3$, 5% HCl and saturated brine. After drying over sodium sulfate, the organic solution was evaporated and the crude product purified by flash chromatography on silica gel using 1% methanol in chloroform. Pooled fractions were evaporated to yield 1.94 g. of N- α -t-butoxycarbonyl-L-phenylalanyl-L-proline benzyl ester as a white solid. The product was characterized by IR and NMR.

Trifluoroacetic acid was added to a cold (0°) solution of the above product (0.937 g., 2.15 mmol) in methylene chloride (20 ml). The reaction mixture was stirred for 1 hour at 0°, after which the methylene chloride and trifluoroacetic acid were evaporated. The residue (1.00 g., 2.15 mmol) and N-methyl-trans-4-acetoxy-L-proline hydrochloride (0.40 g., 2.15 mmol) were suspended in methylene chloride (10 ml) and cooled to 0°. Triethylamine (1.0 ml, 6.5 mmol) was added dropwise followed by 1-hydroxybenzotriazole (0.421 g., 2.74 mmol) and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (0.5 g., 2.62 mmol). The reaction mixture was stirred for 24 hours and allowed to warm to ambient temperature. Methylene chloride (20 ml) was added to the reaction mixture and the organic solution washed sequentially with 5% $NaHCO_3$ and saturated brine. After drying over Na_2SO_4 , the organic solution was evaporated and the resulting crude product, N-methyl-trans-4-acetoxy-L-phenylalanyl-L-proline benzyl ester trifluoroacetate purified by RP-HPLC using a Vydac C-18 one inch column; gradient system: Buffer A, 0.1% TFA/ H_2O ; Buffer B, acetonitrile, 0-100% in 30 min. with UV detection at 220 nm. Pooled fractions were lyophilized to give 0.693 g. of product as a white powder with R_t = 18.97 min. This product was characterized by IR, NMR and MS. The fast atom bombardment (FAB) MS of the powder showed $(M+H)^+$ calc'd. 521.3. Found 522.

A suspension of 10% palladium on carbon (0.203 g.) in water (0.5 ml) was added to a solution of the above product (0.782 g., 1.23 mmol) in methanol (20 ml) in a Parr bottle. The bottle was pressurized to 45 psi with hydrogen and shaken at ambient temperature for 3 hours. The catalyst was removed by filtration through a pad of celite which was washed thoroughly with methanol (50 ml). Evaporation of the methanol afforded 0.547 g. of N-methyl-trans-4-acetoxy-L-prolyl-L-phenylalanyl-L-proline as a colorless oil.

Oxalyl chloride (0.325 ml, 3.73 mmol) was added to a cold (-10°) solution of the oil (0.79 g., 1.83 mmol) in a mixture of acetonitrile (20 ml) and dimethylformamide (1 ml) and the reaction mixture stirred for 30 min. A solution of 1-methyl-4-hydroxypiperidine (0.210 g., 1.83 mmol) in acetonitrile (2 ml) was added to the reaction mixture at -10° and the whole stirred an additional 30 min. at -10° after which the reaction mixture was warmed to ambient temperature during 14 hours. Methylene chloride (50 ml) was added to the reaction mixture which was washed successively with 5% $NaHCO_3$, saturated brine and water. The organic layer was dried over Na_2SO_4 and evaporated in vacuo to give a yellow oil which was purified by RP-HPLC using a Vydac C-18 one-inch column; gradient system: Buffer A 0.1% TFA/ H_2O ; Buffer B, acetonitrile, 0-100% in 30 min. with UV detection at 220 nm. Pooled fractions were lyophilized to afford the tris-trifluoroacetate salt of 1-methyl-4-piperidyloxy ester of N-methyl-trans-4-acetoxy-L-prolyl-L-phenylalanyl-L-proline as a syrup with R_t = 14.50 min. This product was characterized by IR, NMR and MS. The fast atom bombardment (FAB) MS showed $(M+H)^+$ calc'd; 528.7. Found 529.0. Anal. calc'd. for $C_{28}H_{40}N_4O_6 \times 3CF_3CO_2H$: C, 46.90; H, 4.98; N, 6.43. Found: C, 46.75; H, 5.03; N, 6.29.

A solution of the above product (0.266 g., 0.36 mmol) in methanol (10 ml) containing methyl iodide (3.0 ml, 48.2 mmol) was treated with sodium bicarbonate (0.344 g., 4.1 mmol) and the resulting suspension stirred at ambient temperature for 24 hours. The excess methyl iodide and methanol were evaporated in vacuo and the residue treated with acetone and filtered through celite to remove inorganic salts. The acetone was evaporated and the residue dissolved in 0.5% aqueous TFA and purified by RP-HPLC as described above. Lyophilization of pooled fractions gave the tetrakis-trifluoroacetate salt of 1,1-dimethyl-4-piperidyloxy ester of N,N-dimethyl-trans-4-acetoxy-L-prolyl-L-phenylalanyl-L-proline monohydrate as a white powder (0.124 g.) with a R_t = 13.25 min. This product was characterized by IR, NMR and MS. Analysis calculated for $C_{30}H_{46}N_4O_6 \times 2CF_3CO_2H \times H_2O$: C, 44.28; H, 4.89; N, 5.44. Found: C, 44.15; H, 4.67; N, 5.35.

Example 4

Bis-trifluoroacetate salt of L-prolyl-L-(γ -dimethyl)diaminobutyryl-L-phenylalanine methyl ester.

5 Copper (II) carbonate (3.7 g., 16.7 mmol) was added in portions to a cold (0°) solution of L-2,4-diaminobutyric acid dihydrochloride (1.0 g., 6.17 mmol) in water (43 ml), and the resulting aqua suspension was refluxed for 2.5 hours. The reaction mixture was cooled to room temperature, filtered, the solid washed with water (25 ml) and the combined deep blue filtrate and washes cooled to 0°. Magnesium oxide (1.24 g., 31.0 mmol) was added, followed by dropwise addition of benzyl chloroformate (1.4 ml, 9.84 mmol). The
10 reaction mixture was warmed to ambient temperature and stirred for 16 hours. The reaction mixture was filtered and the lavender precipitate washed with diethyl ether (25 ml) and air dried to give 3.17 g. of a lavender solid which was dissolved in cold (0°) 2N HCl (45 ml) and treated dropwise with a solution of sodium sulfide nonahydrate (1.70 g., 7.08 mmol) in water (130 ml). A black solid precipitated (CuS) with the evolution of H₂S. After stirring the black suspension at 0° for 30 minutes, the copper (II) sulfide was
15 removed by filtration. The colorless filtrate was allowed to stand for 1 hour and then the pH adjusted to 4. The white suspension was filtered and the white solid washed with ethanol, then diethyl ether and air dried to yield L-(N- γ -benzyloxycarbonyl)diaminobutyric acid hydrochloride (0.748 g), R_t = 15.50 min.

A suspension of the above product (1.00 g., 3.47 mmol) in dioxane (6 ml) and water (5 ml) was stirred at room temperature during the addition of sodium carbonate (0.99 g., 9.42 mmol) and di-*t*-butyl dicarbonate (0.91 g., 4.17 mmol). Stirring was continued at ambient temperature for 22 hours after which the reaction
20 mixture was evaporated and the residue dissolved in sodium bicarbonate. Following extraction with ethyl acetate to remove excess di-*t*-butyl dicarbonate, the aqueous solution was acidified to pH 6 with 12 M HCl. The product was extracted into ethyl acetate and the resulting organic solution washed with brine and dried over MgSO₄. Filtration of the MgSO₄ and evaporation afforded 1.03 g. of N- α -*t*-butoxycarbonyl-L-(N- γ -benzyloxycarbonyl)diaminobutyric acid as a white foam with an R_t = 19.65 min.
25

Triethylamine (0.65 ml, 4.65 mmol), 1-hydroxybenzotriazole (0.63 g., 4.65 mmol), N- α -*t*-butoxycarbonyl-L-(N- γ -benzyloxycarbonyl)diaminobutyric acid (1.24 g., 3.39 mmol) produced above and dicyclohexylcarbodiimide (0.96 g., 4.65 mmol) were sequentially added to a solution of L-phenylalanine methyl ester hydrochloride (1.0 g., 4.65 mmol) in methylene chloride (7 ml) and dimethylformamide (14 ml) at 0°. Dimethylformamide (10 ml) was added to facilitate stirring and the reaction mixture was stirred at 0° for 2
30 hours, then at ambient temperature for 7 hours. The reaction mixture was filtered and the dicyclohexyl urea washed with tetrahydrofuran. The organic filtrate and washes were combined and evaporated in vacuo. The residue was dissolved in ethyl acetate (300 ml) and washed successively with 5% NaHCO₃, 1M HCl, 5% NaHCO₃ and brine. Evaporation of the ethyl acetate gave N- α -*t*-butoxycarbonyl-(N- γ -benzyloxycarbonyl)-L-diaminobutyryl-L-phenylalanine methyl ester which was washed with a little tetrahydrofuran (25 ml) and air
35 dried to yield 1.59 g. of a white solid with R_t = 25.59 min.

Trifluoroacetic acid (20 ml, 260.0 mmol) was added to a solution of the above product (1.30 g., 2.53 mmol) in methylene chloride (50 ml) at 0° and the reaction mixture was stirred at 0° for 40 min. The methylene chloride and excess trifluoroacetic acid were evaporated to leave a syrup with an R_t = 18.04
40 min which was dissolved in methylene chloride (4 ml), combined with dimethylformamide (10 ml) and cooled to 0°. Triethylamine (1.4 ml, 10.04 mmol) was added followed by 1-hydroxybenzotriazole (0.33 g., 2.47 mmol), N- α -*t*-butoxycarbonyl-L-proline (0.53g., 2.47 mmol) and dicyclohexylcarbodiimide (0.51 g., 2.47 mmol). The reaction mixture was stirred for 8 hours during which time it was allowed to warm to ambient temperature. The reaction mixture was filtered and the dicyclohexyl urea was washed with tetrahydrofuran
45 (25 ml). The combined organic washes and filtrate were concentrated in vacuo to a small volume. Ethyl acetate (150 ml) was added and the organic solution washed successively with 5% NaHCO₃, 1M HCl, 5% NaHCO₃ and brine. The ethyl acetate was dried over MgSO₄, filtered and evaporated to give 1.37 g. of N- α -*t*-butoxycarbonyl-L-prolyl-L-(N- γ -benzyloxycarbonyl)diaminobutyryl-L-phenylalanine methyl ester as a light tan gel with an R_t = 23.34 min. The product was characterized by IR, NMR and MS. The fast atom
50 bombardment (FAB) MS showed (M+H)⁺ at m/z cal'd: 610.7. Found: 611.

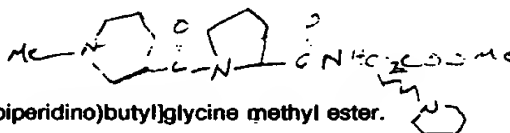
A solution of the above methyl ester (1.37 g., 2.25 mmol) in methanol (200 ml) was charged in a Parr bottle. A suspension of 10% palladium on carbon (0.06 g.) in water (7 ml) was added and the bottle pressurized to 48 psi with hydrogen. The reaction mixture was shaken for 6 1/2 hours at ambient
55 temperature. The catalyst was removed by filtration through a celite pad which was washed thoroughly with methanol (50 ml). The combined filtrate and washes were evaporated in vacuo to leave 1.01 g. of a colorless syrup which has an R_t = 19.83 min. together with a few trace impurities.

A solution of the above product, without purification (0.050 g., 1.05 mmol) in ethanol (50 ml) was charged in a Parr bottle. To the solution was added 37% aqueous formaldehyde (3.2 ml) followed by a

suspension of 10% palladium on carbon (0.65 g.) in water (3 ml). The bottle was pressurized to 45 psi with hydrogen and shaken at ambient temperature for 15 hours. The catalyst was removed by filtration through a pad of celite which was washed thoroughly with methanol (25 ml). The combined filtrate and washes were evaporated in vacuo to leave a syrupy solid which was taken up in methanol (25 ml) and filtered to remove paraformaldehyde. Evaporation of the methanol produced a residue which was purified by flash chromatography on silica gel using ethyl acetate-hexane-methanol (10 : 1 : 6). Pooled fractions were combined and evaporated to leave 0.23 g. of N- α -t-butyloxycarbonyl-L-prolyl-L-(γ -dimethyl)diaminobutyl-L-phenylalanine methyl ester as a syrup with an R_t = 19.11 min. The product was characterized by IR, NMR and MS. The fast atom bombardment (FAB) MS showed (M+H)⁺ at m/z calc'd: 504.6. Found: 505.

Trifluoroacetic acid (3 ml) was added to a solution of the above ester (0.20 g., 0.39 mmol) in methylene chloride (6 ml) and the reaction mixture stirred at 0° for 45 min. The methylene chloride and excess trifluoroacetic acid were evaporated in vacuo. The syrupy residue was dissolved in water (25 ml) and the aqueous solution washed with diethyl ether. Lyophilization yielded a hygroscopic syrup which was purified by RP-HPLC using a Vydac C-18 one inch column; gradient system: Buffer A, 0.1% TFA/H₂O; Buffer B, acetonitrile, 0-100% in 30 min with UV detection at 220 nm. Pooled fractions were lyophilized to give 0.11 g. of L-prolyl-L-(γ -dimethyl)diaminobutyl-L-phenylalanine methyl ester bis-trifluoroacetate as a glass with an R_t = 13.95 min. The product was characterized by IR, NMR and MS. The fast atom bombardment (FAB) MS shows (M+H)⁺ at m/z calc'd.: 404.5. Found: 405. Anal. calc'd for C₂₁H₃₂N₄O₄ x 2CF₃CO₂H x 2H₂O: C, 44.91; H, 5.73; N, 8.38. Found: C, 44.95; H, 5.41; N, 8.18.

Example 5



Bis-trifluoroacetate of N-methyl-L-nipecotyl-L-prolyl- α -S-[4-(1-piperidino)butyl]glycine methyl ester.

L-Nipecotic acid (1.29 g., 10 mmol), 10% Pd/C catalyst (0.76 g), and 37% aqueous formaldehyde (1 ml, 13.3 mmol) were dissolved in methanol (35 ml). The mixture was placed in a Parr hydrogenation apparatus under H₂ (45 psi) and shaken for 12 hours. The reaction mixture was flushed with N₂, filtered through celite and concentrated on a rotary evaporator to give 1.29 g. of N-methyl-L-nipecotic acid, structure verified by NMR.

L-(N- ϵ -benzyloxycarbonyl)lysine methyl ester hydrochloride (3.31 g., 10 mmol), N- α -t-butyloxycarbonyl-L-proline (2.15 g., 10 mmol), 1-hydroxybenzotriazole (2.01 g., 15 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC x HCl) (2.66 g., 15 mmol) and triethylamine (3.6 ml, 25 mmol) were dissolved in CH₂Cl₂ (25 ml) in a cooled ice water bath. The reaction mixture was stirred for 24 hours during which time it warmed to room temperature. Methylene chloride (50 ml) was added to the reaction mixture and the solution washed twice with dilute HCl and 5% NaHCO₃. The organic layer was dried over MgSO₄ and concentrated in vacuo to give a residue of 4.6 g. of N- α -t-butyloxycarbonyl-L-prolyl-L-(N- ϵ -benzyloxycarbonyl)lysine methyl ester. HPLC showed a single peak at R_t = 22.7 min.

The above ester (4.6 g., 9.3 mmol) was dissolved in CH₂Cl₂ (20 ml) and cooled in an ice water bath. Trifluoroacetic acid (20 ml) was then added and the reaction mixture stirred for 1 hour at 0°. The mixture was concentrated in vacuo, Et₂O (20 ml) was added and the reaction mixture was concentrated again to give 4.56 g. of white solid. HPLC showed one peak at R_t = 17.02 min.

The resulting product (4.56 g., 9.0 mmol) was dissolved in CH₂Cl₂ (50 ml), the N-methyl-L-nipecotic acid prepared above (9.29 g., 9.0 mmol) added thereto and the solution cooled to 0°. EDC (1.5 g., 15.0 mmol), 1-hydroxybenzotriazole (1.23 g., 9.0 mmol) and NEt₃ (2.5 ml, 18.0 mmol) were added and the reaction mixture stirred for 17 hours during which time it warmed to room temperature. The mixture was washed with 5% NaHCO₃, brine and the solvent concentrated on a rotary evaporator. The resultant solid was taken up in CH₂Cl₂ and purified by silica gel chromatography using 10% MeOH in CHCl₃ as eluent. Pooled fractions were concentrated to give 1.8 g. of N-methyl-L-nipecotyl-L-prolyl-L-(N- ϵ -benzyloxycarbonyl)lysine methyl ester. HPLC showed one peak at R_t = 16.94 min.

The above ester (930 mg, 1.8 mmol) was dissolved in methanol (100 ml) and 10% Pd/C (300 mg) in water (2 ml) plus TFA (0.3 ml) were added. The mixture was placed in a Parr hydrogenation apparatus under H₂ (50 psi) and shaken for 4 hours. The reaction mixture was flushed with N₂, filtered through celite and concentrated in vacuo. The resulting gummy solid was triturated with Et₂O, and placed under vacuum over night to give 894 mg white solid which was purified using RP-HPLC, as described in Example 1, to give 550 mg of powder after lyophilization. Analytical HPLC showed one peak at R_t = 13.36 min.

The peptide formed above, (246 mg, 0.48 mmol) was dissolved in methanol (50 ml) and combined with 25% aqueous glutaric dialdehyde (0.4 ml, 1 mmol). HEPES at pH 6 was then added, followed by NaBH₃CN (343 mg, 5 mmol) and the reaction mixture was stirred at room temperature for 4 hours. The solvent was

evaporated on a rotary evaporator and the resulting solid was taken up in CH_2Cl_2 (100 ml). The solution was washed twice with 5% NaHCO_3 and extracted into $\text{TFA}/\text{H}_2\text{O}$ (100 ml). The aqueous layer was washed with Et_2O and then lyophilized to give 285 mg of solid.

The product was purified on RP-HPLC as described in Example 1, and the pooled fractions lyophilized to give 165 mg of solid. Anal. calc'd for $\text{C}_{24}\text{H}_{42}\text{N}_4\text{O}_4 \times 2\text{CF}_3\text{CO}_2\text{H} \times \text{H}_2\text{O}$: C, 48.27; H, 6.66; N, 8.05. Found: C, 48.39; H, 6.66; N, 7.59. These results are consistent with the bis-trifluoroacetate salt of N-methyl-L-nipecotyl-L-prolyl- α -S-[4-(1-piperidino)butyl]glycine methyl ester bis-trifluoroacetate.

Example 6

Methyl ester of N- α -t-butyloxycarbonyl- α -S-[4-(1-piperidino)butyl]glycyl-L-phenylalanyl- α -S-[4-(1-piperidino)butyl]glycine-tris-trifluoroacetate.

A stirred solution of (N- ϵ -benzyloxycarbonyl)-L-lysine hydrochloride methyl ester (2.21 g., 6.7 mmol), N- α -t-butyloxycarbonyl-L-phenylalanine (2.0 g., 6.7 mmol), 1-hydroxybenzotriazole (HOBt) (1.81 g., 13.4 mmol), and 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (WSCl x HCl) (2.57 g., 13.4 mmol) in CH_2Cl_2 (30 ml), was cooled to 5° and triethylamine (2.19 g., 21.6 mmol) added via syringe at constant temperature. Stirring was continued in the cold for 2 hours and then the reaction mixture was allowed to come to room temperature. After stirring for 18 hours, the reaction mixture was washed with saturated NaHCO_3 solution, 1M HCl and dried over magnesium sulfate. After removal of solvent in vacuo, the residue was triturated with ether/hexane to yield the methyl ester of N- α -t-butyloxycarbonyl-L-phenylalanyl-L-(N- ϵ -benzyloxycarbonyl)lysine as a white solid (3.2 g.). The structure was supported by NMR and IR.

The ester formed above (0.83 g., 1.57 mmol) was dissolved in methylene chloride (20 ml), cooled to 5° and treated with trifluoroacetic acid (TFA) (5 ml). The reaction mixture was stirred at room temperature for 1 hour, concentrated in vacuo and the residue triturated with diethyl ether to give an amorphous solid (0.85 g.). The NMR was consistent with the desired structure.

The above product (0.85 g., 1.53 mmol) in methylene chloride (10 ml) was cooled in an ice bath and treated with triethylamine (0.15 g., 1.53 mmol). After 30 minutes, the mixture was treated with N- α -t-butyloxycarbonyl-(N- ϵ -benzyloxycarbonyl)lysine (0.60 g., 1.53 mmol), 1-hydroxybenzotriazole (0.21 g., 1.53 mmol), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (WSCl x HCl) (0.29 g., 1.53 mmol). Triethylamine (0.15 g., 1.53 mmol) was added via syringe at constant temperature. Stirring was continued in the cold bath for 2 hours after which the reaction mixture was allowed to come to room temperature. After stirring for 18 hours, the reaction mixture was washed with saturated NaHCO_3 solution, 1M HCl, and brine, and dried over magnesium sulfate. After removal of solvent in vacuo, the residue was triturated with ether/hexane to afford the product as a white amorphous solid (0.96 g.). The solid was dissolved in a minimum amount of diethyl ether and loaded onto a column of silica gel (50 X 5 cm). The column was eluted with diethyl ether/hexane (1/1) to isolate a less polar impurity followed by ether/hexane (4/1) to afford the fully protected tripeptide derivative. The pooled fractions were evaporated in vacuo to give the methyl ester of N- α -t-butyloxycarbonyl-(N- ϵ -benzyloxycarbonyl)-L-lysyl-L-phenylalanyl-L-(N- ϵ -benzyloxycarbonyl)-lysine as a white solid, confirmed by NMR.

To a solution of the above methyl ester (0.93 g., 1.15 mmol) in methanol (40 ml) and water (4 ml) contained in a Parr hydrogenation bottle was added 0.30 g. of 10% palladium or carbon and the resulting suspension placed under hydrogen at 50 psi and shaken for 21 hours. The reaction mixture was filtered through celite and the filtrate concentrated to give the methyl ester of N- α -t-butyloxycarbonyl-L-lysyl-L-phenylalanyl-L-lysine bis-trifluoroacetate salt which was purified by RP-HPLC employing an 0.1% aqueous trifluoroacetic acid/acetonitrile linear gradient. The pooled fractions were lyophilized to give the product as a hygroscopic white solid (0.67 g.). The ^1H -NMR was consistent with the desired structure.

To a stirred solution of the above product (300 mg, 0.39 mmol) in acetonitrile (10 ml) was added 10 ml of 100 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES) buffer and 6 drops of bromocresol green indicator, followed by 0.2 ml glutaric dialdehyde (50% wt. aqueous solution, 1.10 mmol) and sodium borohydride (400 mg., 10.6 mmol). The reaction mixture was stirred at room temperature for 3 hours maintaining the pH above 5.4. After removal of solvent in vacuo, the residue was dissolved in 50 ml of methylene chloride and washed several times with 5% NaHCO_3 . The organic phase was dried over MgSO_4 , filtered and concentrated and the residue purified by RP-HPLC using a linear gradient of 0.1% aqueous trifluoroacetic acid/acetonitrile. The pooled fractions were lyophilized to give the methyl ester of N- α -t-butyloxycarbonyl- α -S-[4-(1-piperidino)butyl]glycyl-L-phenylalanyl- α -S-[4-(1-piperidino)butyl] glycine tris-trifluoroacetate as a white solid (95 mg). The ^1H -NMR was consistent with the desired structure. FAB mass

spec. $[M + H]^+ = 672$. Anal. calcd. for $C_{37}H_{61}N_5O_6 \times 3CF_3CO_2H$: C, 50.93; H, 6.36; N, 6.91. Found: C, 50.81; H, 6.60; N, 7.33.

Example 7

The following bioassay methodologies were used to demonstrate the neuromuscular junction blocking activity of the compounds of the invention. The relaxant properties of compounds with this pharmacologic mechanism could be used during surgical anesthesia to facilitate endotracheal intubation and retraction of muscle groups as required to expedite access to various body cavities. Each of these tests extended the knowledge of the clinical potential of the subject compounds. In instances where the compounds of this invention were not subjected to analysis in a specific test, it is possible to estimate such activity based on known relationships to other clinically available drugs which had been tested.

The first step, in mice, establishes a preliminary estimate of the potency and efficacy of the compounds. The animals were placed on a screen, inclined 45° to the horizontal. Effective doses caused the mice to lose their grip and slide down the inclined screen. The dose in mg/kg of body weight required to inhibit grip strength in 100% of the mice tested in a dosage group is reported.

The type of muscle relaxation produced by the test compounds was then determined by injection into chicks. Compounds which cause competitive blockage of post-synaptic acetylcholine receptors, i.e. non-depolarizing drugs, produce a flaccid paralysis in the chicks whereas drugs which cause depolarization of the post-synaptic muscle membrane produce a rigid paralysis. Only those compounds shown by this test to be nondepolarizing are tested further. This test established that the subject compounds are nondepolarizing muscle relaxants.

The rabbit paw twitch analysis was used to demonstrate the rate of onset and duration and to confirm the range of potency of test compounds. In a large series of similar relaxants, the rabbit dose is typically 125% of that in mice. The mechanism of action was also confirmed in this test by observing train-of-four and tetanus fade, post-tetanic potentiation of single twitches and administration of the anticholinesterase drug neostigmine which reverses the relaxation. Reversibility, rapid onset and short duration are important factors to the anesthesiologist.

In Table I, the doses of the compounds of the invention are shown relative to doses of clinically available drugs. Clinically, 0.1 to 0.14 mg/kg of vecuronium has been used for endotracheal intubation, while 0.010 mg/kg is used for maintenance of relaxation. Therefore, as an estimate of the range of possible dosages which might be employed for the subject compounds, the ED₉₀ would be doubled as an estimate for an intubating dose, while a dose 20 to 25% of the ED₉₀ dose might be required for maintenance bolus doses. The clinical dose range might be between 29% to 200% of the estimated ED₉₀.

Table I

Neuromuscular Junction Blocking Activity (ED₉₀ in mg/kg)

<u>Drug</u>	<u>Mouse</u>	<u>Rabbit</u>
vecuronium	0.025	0.020
atracurium	0.631	0.050
Compound A	10.000	9.920
Compound B	16.000	31.26
Compound C	16.000	19.80
Compound D	10.000	N/A
Compound E	3.981	N/A

The detailed pharmacologies in rabbits of two of the subject compounds are presented in Table II. The following is a brief description of the methodologies used in rabbits to describe neuromuscular blocking activity of the subject compounds. A more detailed description of these methods is presented in "Microcomputer Use in Measuring Onset, Duration, and Recovery from Non-Depolarizing Skeletal Muscle Relaxants in Rabbits", P.D. Thut et al., Drug development Research 5:182, 1985.

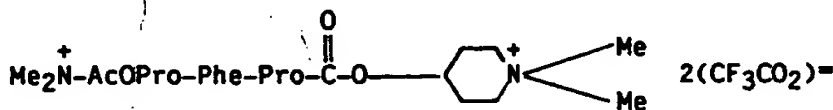
Male New Zealand white rabbits weighing between 2.5 and 3.4 kg were anesthetized with pentobarbital (30 mg/kg) and placed on their backs upon a $40^\circ C$ water filled temperature regulation pad. Following tracheostomy, the lungs were mechanically ventilated at 28 breaths per minute with room air, using an open

system delivering 200 ml/stroke. This ventilation maintained pCO₂ at 38 mmHg and pO₂ at 85 mmHg. Direct arterial blood pressure was measured from the right common carotid artery. The test compounds were administered through a cannula placed in the marginal ear vein. Each foreleg was taped to a cushioned plate held in a femur clamp attached to the spinal board rack. The left central digit of each paw was connected to a force displacement transducer for measurement of muscle tension. Nerve stimulation was provided by pairs of pin electrodes placed on both sides of the ulnar nerve at the elbow of both forearms. The right ulnar nerve was stimulated at 1 Hz, 1 pps for .5 msec duration. The left ulnar nerve was similarly stimulated, every 15 seconds, with addition of interspersed trains-of-four and tetanizing stimuli. The parameters reported in Table II are: potency (ED₉₀), which is the dose required to depress twitch tension to 10% of its control value; onset (T₈₅%), which is the time from injection until 85% of the maximal drug effect is achieved; duration, which is the time from injection until the train-of-four has recovered to 75%; blood pressure (BP), which is the percentage change of pre-drug blood pressure; and heart rate (HR), which is the percentage change from pre-drug heart rate.

Table II

Rabbit Paw Twitch Equi-efficacious Dose Data					
Compound	ED ₉₀ (mg/kg)	T ₈₅ % (seconds)	Duration (minutes)	BP (% change)	HR (% change)
atracurium	0.05	126.80	14.30	- 3.40	- 1.00
vecuronium	0.02	97.30	16.80	1.10	- 9.90
pancuronium	0.02	147.50	35.50	2.70	0.00
Compound A	9.92	27.40	16.10	- 22.00	- 7.90
compound B	31.26	24.40	22.00	- 18.83	8.79

In Tables I and II, the designated compounds are as follows:

Compound ACompound B

Boc-Lys(ε-N-Me₃)-Phe-Lys(ε-N-Me₃)OMe 2(CF₃CO₂) = (all AA D-form)

Compound C

Me₃N-Phe-Leu-Lys(ε-N-Me₃)OMe 2(CF₃CO₂) = (all AA D-form)

Compound D

Boc-Lys(ε-N-Me₃)-2-Naphthala-Lys(ε-N-Me₃)OMe 2(CF₃CO₂) =

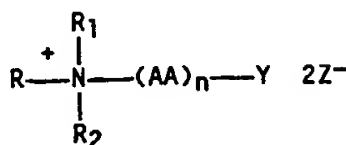
Compound E

Boc-Lys(ε-Me-N(CH₂)₅)-Phe-Lys(ε-Me-N(CH₂)₅)OMe 2(CF₃CO₂) =

The results in the Tables show that the subject compounds, while not as potent as those utilized for comparison, are advantageous in that they have a significantly shorter onset of activity.

Claims

1. A quaternary polypeptide selected from those represented by the formula:

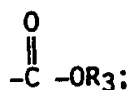


wherein:

n is 3, 4 or 5;

R is lower alkyl; and

R₁ and R₂ are independently selected from the group consisting of lower alkyl, allyl, propargyl, aryl lower alkyl, cyclo-lower alkyl lower alkyl and



or R plus one or both of R₁ and R₂, together with the nitrogen to which they are attached form a heterocyclic ring having 5 to 7 member atoms;

at least one AA is a basic amino acid selected from the group consisting of DAB(γ-N-RR₁R₂), Orn-(δ-N-RR₁R₂) and Lys(ε-N-RR₁R₂);

at least one AA is a lipophilic amino acid selected from the group consisting of Ala, Val, Leu, Ile, Phe, Tyr, Pro, Tic, 1-Naphthala, 2-Naphthala, homo-Phe, AcOPro and Glu(γ-Me), or two of the lipophilic AA are cystine;

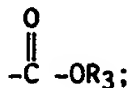
Y is -NH₂ or -OR₄;

R₃ is selected from the group consisting of t-butyl, benzyl or fluorenylmethyl;

R₄ is selected from the group consisting of lower alkyl, aryl lower alkyl and N-(lower alkyl)-piperidyl lower alkyl with the proviso that, wherein R₄ is N-(lower alkyl)-piperidyl lower alkyl, a basic amino acid is not present; and

Z⁻ is a pharmaceutically acceptable anion.

2. A polypeptide in accordance with Claim 1, wherein R₁ and R₂ are selected from the group consisting of lower alkyl, allyl,

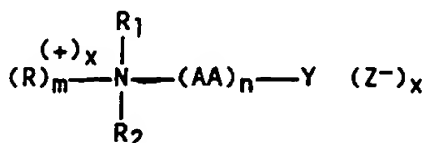


and cyclo lower alkyl lower alkyl.

3. A polypeptide in accordance with Claim 1, wherein R plus one or both of R₁ and R₂, plus the nitrogen to which they are attached, form a heterocyclic ring selected from the group consisting of pyrrolidine, piperidine, hexamethyleneimine, piperazine and morpholine.

4. A polypeptide in accordance with Claim 1, wherein said lipophilic amino acid is selected from the group consisting of Phe, Tyr, Leu and 2-Naphthala, R_3 is t-butyl, benzyl or fluorenylmethyl; Y is $-NH_2$ or $-OR_4$ and R_4 is N-(lower alkyl)-piperidiny lower alkyl.

5. A pharmaceutical composition intended as a muscle relaxant comprising a pharmaceutical carrier suitable for parenteral administration and a polypeptide selected from those represented by the formula:



and pharmaceutically acceptable salts thereof,
wherein:

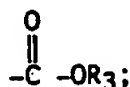
m is 0 or 1;

n is 3, 4 or 5;

x is 0 or 2;

R is lower alkyl; and

R_1 and R_2 are independently selected from the group consisting of lower alkyl, allyl, propargyl, aryl lower alkyl, cyclo-lower alkyl lower alkyl and



or R plus one or both of R_1 and R_2 , together with the nitrogen to which they are attached form a heterocyclic ring having 5 to 7 member atoms;

at least one AA is a basic amino acid selected from the group consisting of DAB, Orn, Lys, Arg, homo-Arg, DAB(γ -N-(R) $_m$ R $_1$ R $_2$), Orn(δ -N-(R) $_m$ R $_1$ R $_2$) and Lys(ϵ -N-(R) $_m$ R $_1$ R $_2$);

at least one AA is a lipophilic amino acid selected from the group consisting of Ala, Val, Leu, Ile, Phe, Tyr, Pro, Tic, 1-Naphthala, 2-Naphthala, homo-Phe, AcOPro and Glu(γ -Me), or two of the lipophilic AA are cystine;

Y is $-NH_2$ or $-OR_4$;

Z^- is a pharmaceutically acceptable anion;

R_3 is selected from the group consisting of t-butyl, benzyl or fluorenylmethyl;

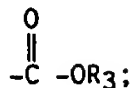
R_4 is selected from the group consisting of lower alkyl, aryl lower alkyl and N-(lower alkyl)-piperidyl lower alkyl, with the proviso that, wherein R_4 is N-(lower alkyl)-piperidyl lower alkyl, a basic amino acid is not present; and

wherein m is 1, x is 2, said basic amino acid is selected from the group consisting of DAB(γ -N-(R) $_m$ R $_1$ R $_2$), Orn(δ -N-(R) $_m$ R $_1$ R $_2$) and Lys(ϵ -N-(R) $_m$ R $_1$ R $_2$) or R_4 is N-(lower alkyl)-piperidyl lower alkyl, and said basic amino acid is not present; and

wherein m is 0, pharmaceutically acceptable acid addition salts thereof.

6. A composition in accordance with Claim 5, wherein R_1 and R_2 are independently selected from the

group consisting of hydrogen, lower alkyl, allyl and



- 5
- 10 7. A composition in accordance with Claim 5, wherein R plus one or both of R₁ and R₂, together with the nitrogen to which they are attached, form a heterocyclic ring selected from the group consisting of pyrroli- dine, piperidine, hexamethyleneimine, piperazine and morpholine.
- 15 8. A composition in accordance with Claim 5, wherein said basic amino acid is selected from the group consisting of
- DAB(γ -N-(R)_mR₁R₂), Orn(δ -N(R)_mR₁R₂),
Lys(ϵ -N-(R)_mR₁R₂), Arg and homo-Arg,
- 20 said lipophilic amino acid is selected from the group consisting of Phe, Tyr, Leu, 2-Naphthala and Tic.
9. A composition in accordance with Claim 5, wherein R₃ is t-butyl and R₄ is lower alkyl or N-(lower alkyl)-piperidyl lower alkyl.
- 25 10. A composition in accordance with Claim 5, wherein said composition contains a member selected from the group consisting of:
- (a) the methyl ester of N- α -t-butyloxycarbonyl- α -S-[4-[1-(1-methyl)piperidinium]butyl]glycyl-L-phenylalanyl- α -S-[4-[1-(methyl) piperidinium]butyl]glycine;
- 30 (b) the 1,1,-Dimethyl-4-piperidyloxy ester of N,N-Dimethyl-trans-4-acetoxy-L-prolyl-L-phenylalanyl-L-proline;
- (c) the methyl ester of N- α -t-butyloxycarbonyl-L-(N- ϵ -trimethyl) lysyl-L-phenylalanyl-L-(N- ϵ -trimethyl)-lysine;
- (d) the methyl ester of N- α -t-butyloxycarbonyl-L-(N- ϵ -trimethyl) lysyl-L-2-naphthylalanyl-L-(N- ϵ -trimethyl)lysine;
- 35 (e) the methyl ester of N- α -t-butyloxycarbonyl- α -S-[4-(1-piperidino)butyl]glycyl-L-phenylalanyl- α -S-[4-(1-piperidino)butyl]glycine; and
- (f) the methyl ester of N- α -t-butyloxycarbonyl-L-lysyl-1,2,3,4-tetrahydroisoquinoliny-L-lysine.
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- 55